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2 Assessment of Age-Related Changes in Pediatric Gastrointestinal Solubility

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Abstract:

Purpose: Compound solubility serves as a surrogate indicator of oral biopharmaceutical performance. Between infancy and adulthood, marked compositional changes in gastrointestinal (GI) fluids occur. This study serves to assess how developmental changes in GI fluid composition affects compound solubility.

Methods: Solubility assessments were conducted *in vitro* using biorelevant media reflective of age-specific pediatric cohorts (i.e. neonates and infants). Previously published adult media (i.e. FaSSGF, FeSSGF, FaSSIF.v2, and FeSSIF.v2) were employed as references for pediatric media development.

Investigations assessing age-specific changes in GI fluid parameters (i.e. pepsin, bile acids, pH, osmolality, etc.) were collected from the literature and served to define the composition of neonatal and infant media. Solubility assessments at 37°C were conducted for seven BCS Class II compounds within the developed pediatric and reference adult media.

Results: For six of the seven compounds investigated, solubility fell outside an 80-125% range from adult values in at least one of the developed pediatric media. This result indicates a potential for age-related alterations in oral drug performance, especially for compounds whose absorption is delimited by solubility (i.e. BCS Class II).

Conclusion: Developmental changes in GI fluid composition can result in relevant discrepancies in luminal compound solubility between children and adults.

42	Abbreviations:
43	
44	IVIVC – <i>in vitro</i> – <i>in vivo</i> correlations
45	GI – gastrointestinal
46	P-BCS – pediatric biopharmaceutics classification systems
47	BCS - biopharmaceutics classification systems
48	FaSSGF- fasted-state simulated gastric fluid
49	FeSSGF- fed-state simulated gastric fluid
50	FaSSIF- fasted-state simulated intestinal fluid
51	FeSSIF- fed-state simulated intestinal fluid
52	US-FDA - United States Food and Drug Administration
53	NaTc – sodium taurocholate
54	PNA – postnatal age
55	GA – gestational age
56	NEC – necrotizing enterocolitis
57	FFA – free fatty acids
58	
59	

Introduction:

The use of *in vitro* tests to forecast oral drug performance can serve to identify compounds displaying inadequate or unfavorable absorption profiles during early stages of drug development. To facilitate such *in vitro* – *in vivo* correlations (IVIVC), test media utilized should reflect the complex physiochemical nature of human gastrointestinal (GI) fluids. Accordingly, several formulas of biorelevant media have been developed based on the intraluminal conditions of the GI tract in adults (1-3). For immediate release dosage forms, where drug release is expected to occur within the upper region of the GI tract, biorelevant media depicting the stomach and proximal small intestine are typically formulated.

Compared to compendial media, use of biorelevant media within *in vitro* dissolution experiments has been demonstrated to provide IVIVC that better predict oral drug absorption in adults (4, 5). Despite these favorable results, the use of biorelevant media for establishing IVIVC within pediatric populations is contentious. This is because contemporary biorelevant media (1, 2) are formulated based on gastrointestinal conditions of an adult human. Consequently, their applicability towards pediatric populations, who are developmentally distinct in terms of gastrointestinal anatomy/physiology, remains questionable. Of most interest are children belonging to the youngest age groups (i.e. neonates and infants) who display the greatest developmental differences in comparison to adults (6, 7).

In recognition of the potential impact that developmental differences in GI anatomy/physiology can exert on oral drug absorption, a Pediatric Biopharmaceutics Classification System (P-BCS) Working Group was assembled to assess whether a similar classification system as utilized in adults could be developed for children (8). The Biopharmaceutics Classification System (BCS) categorizes drugs based on two properties, aqueous solubility and permeability (9). Accordingly, compounds can be classified as either BCS I (high solubility, high permeability), II (low solubility, high permeability), III (high solubility, low permeability), or IV (low solubility, low permeability). The classification system supports several aspects

of oral drug development in adults, including assessment of generic biowaiver applicability, lead compound selection, and formulation development (8). Based on their findings in a 2012 publication, the P-BCS Working Group concluded that in order to have merit, substantial knowledge gaps with regards to pediatric GI physiology, intestinal permeability, and ontogeny of drug metabolizing enzymes/transporters needs to be addressed prior to establishing of a pediatric-focused BCS (8). To enhance its applicability towards pediatric populations, it is clear that development of a P-BCS would require considerable modification of the current system. Of interest is how developmental changes in GI fluid composition affects compound solubility in relation to adults.

In addition to age-related changes, the composition of GI luminal fluids undergoes positional changes from the stomach to the colon. Changes in composition including bile salt concentration, pH, osmolality, buffer capacity, and presence of fat digestion products, can impart changes in compound specific solubility (10). Therefore, to discern whether relevant differences in luminal solubility exist between pediatrics and adults, quantification of the relationship between age and GI fluid composition is inherently required.

This study serves to assess the impacts of growth and maturation on gastrointestinal solubility. Pediatric biorelevant media representative of the stomach and proximal small intestine were developed based on an assessment of the available literature. Developed pediatric media were utilized to perform solubility assessments for seven BCS Class II compounds. To assess the impact of developmental changes in fluid composition, solubility values were compared between the different age-specific media including media representative of adults.

Methods:

(i)Materials:

Acetic acid (>99.7%), acetonitrile, dapsone, fenofibrate, indomethacin, hydrochloric acid 36.5-38%, methanol, pepsin (from porcine), phenytoin acid and sodium oleate were obtained from Sigma-Aldrich Company Ltd., Dorset, England. Griseofulvin, maleic acid, sodium acetate, sodium chloride, sodium hydroxide, orthophosphoric acid and spironolactone were acquired from Fisher Scientific UK Ltd., Loughborough, England. Ammonium acetate (FSA Laboratory Supplies, Loughborough, UK), carbamazepine (Fagron UK Ltd, Newcastle upon Tyne, England), sodium taurocholate (Prodotti Chimici Alimentari S.P.A., Basaluzzo, Italy), egg lecithin - Lipoid EPCS (Lipoid GmbH, Ludwigshafen, Germany) and glyceryl monooleate - Rylo Mg 19 (Danisco, Brabrand, Denmark) were obtained from the sources specified. Ultra-high-temperature treated whole cow's milk standardized to less than 4% fat was acquired from Sainsbury's, London, England. Two infant formulas manufactured by Cow & Gate, Trowbridge, England were utilized in the study: First Infant Milk (cow's milk-based formula) and Infasoy (soya-based formula). Water was ultra-pure (Milli-Q) laboratory grade. Dialysis tubing (12-14000 Da MWCO) was acquired from Medicell Membranes Ltd., London, England. Equipment utilized in the current investigation included a Buchi R114 Rotavapor (Flawil, Switzerland), a Beckman Coulter J2-MC centrifuge (High Wycombe, England), a Mettler Toledo SevenCompact S210 pH meter (Schwerzenbach, Switzerland), an Advanced Instruments Inc. micro-osmometer Model 3300 (Norwood, MA) and an Agilent Technologies 1200 series HPLC system (Santa Clara, CA): binary pump (G1212A), autosampler (G1329A), thermostatted column compartment (G1316A), and diode array detector (G1315D).

(ii) Media development:

Biorelevant media as characterized by Jantratid et al. (2) were selected as the focal points from which subsequent age-specific media were developed. The authors described four separate media reflective of the physiology of the stomach and proximal small intestine in adults in fasting and fed states:

Fasted-State Simulated Gastric Fluid (FaSSGF), Fed-State Simulated Gastric Fluid (FeSSGF), Fasted-State Simulated Intestinal Fluid v2 (FaSSIF.v2), and Fed-State Simulated Intestinal Fluid v2 (FeSSIF.v2) (Table I). Based on relative differences between adult and pediatric GI physiology, components of the reference adult media were modified to generate age-specific media.

Investigations assessing developmental changes in gastrointestinal fluid composition were collected from the literature. For studies where information was displayed graphically, data was quantified using GetData Graph Digitizer (v2.26). Dependent on the specific media being formulated (i.e. FaSSGF), information pertaining to different physiological parameters were required:

- (a) FaSSGF – pepsin concentrations, pH, osmolality, and bile salt/lecithin concentrations
- (b) FeSSGF – feed type(i.e. cow's milk-based vs. soy-based formula), pH, osmolality, and buffering capacity
- (c) FaSSIF – pH, bile salt/lecithin concentrations, osmolality, and buffering capacity
- (d) FeSSIF – pH, osmolality, bile salt/lecithin concentrations, fat digestion products, and buffering capacity

Parameters values were compiled and, where suitable, graphically displayed as a function of age as an initial evaluation. If changes in GI fluid parameters between pediatric age groups and adults were noted, differences were computed based on a simplistic measure, the arithmetic mean. As the propensity of developmental effects were expected to be most prominent within the earliest stages of life, media reflective of the following age groups were formulated: neonates (0 – 28 days) and infants (1 – 12 months). When data pertaining to specific parameters were unavailable in children, either a default value representative of adult media or an inference based on current physiological knowledge was adopted.

Based on this analysis, biorelevant media reflective of pediatric physiology were defined. Media preparation was conducted using the methods depicted in Jantratid et al (2). Measures of osmolality and

pH were instituted to ensure prepared media conformed to desired values. Osmolality was measured using freezing point depression (Micro-osmometer - Advanced Instruments Inc.). Discrepancies between measured and desired osmolality were corrected by adjusting media sodium chloride concentrations as described in the literature (2). Media pH was titrated using 1M HCl or 1M NaOH, if necessary. Buffering capacity of pediatric formula (cow's milk-based and soy-based) was determined based on the methodology presented by Hentges et al (11). Values presented represent the amount of acid or base (i.e. mEq) required to induce of pH change of 1 unit per litre of formula (12).

(iii) Solubility Assessments:

(a) Compound Selection

Solubility assessments were conducted using BCS class II compounds. Compounds were further restricted to include only those with documented usages, including investigational uses, in both children and adults. Based on the above criteria, seven compounds were selected including carbamazepine, dapson, fenofibrate, griseofulvin, indomethacin, phenytoin and spironolactone. Compound physicochemical properties are displayed in Table II.

(b) Solubility Experiments

Compound specific solubility assessments were conducted in each of the developed pediatric media as well as in the reference adult media to assess for age-related differences. Experiments were conducted within a shaking water bath set to 37°C and 200 strokes/min.

Compound specific solubility values in aqueous-based media (FaSSGF, FaSSIF and FeSSIF) were determined based on the following procedure. A mass of solid (powdered) compound to saturate 10 mL

of biorelevant media was added to borosilicate glass tubes. Next, 10 mL of freshly prepared age-specific media (pediatric or adult) was added. Tubes were covered with parafilm and placed in the shaking water bath. Solubility assessments for all compounds, with the exception of fenofibrate, were conducted following a 24 hour dwell period. For fenofibrate, previous investigations have employed longer dwell periods (i.e. 48-72 hours) in order to achieve equilibrium solubility (13, 14). Correspondingly, a dwell period of 72 hours was utilized in this investigation. Saturated media samples were filtered through 0.45 μ m regenerated cellulose filters and diluted with fresh media prior to assessment. HPLC-UV was utilized to quantify solubility. Analytical HPLC procedures were based on modifications of methods depicted in the literature and are denoted in Table III. Solubility assessments were conducted in triplicate for each test media (pediatric and adult). Calibration curves were constructed using five standard concentrations. Standards were formulated as mobile phase dilutions of a concentrated stock solution consisting of compound dissolved in an organic solvent (i.e. methanol). All dilutions were conducted using volumetric glassware.

Due to the addition of either milk or infant formula, fed-state gastric media exists as a complex multiphase system (15). Proteins within the media deter direct filtration of samples through 0.45 μ m filters. As a result, the investigation utilized equilibrium dialysis to assess compound solubility within all fed-state gastric media, which negated the need for sample filtration to remove excess drug. To ensure restrictions in the rate of membrane permeation did not delimit solubility determinations, samples were permitted to dwell for an additional 24 hours compared to aqueous-based media. A mass of solid compound required to saturate 25 mL of media was added to separate 50 mL plastic centrifuge tubes. Twenty mL of freshly prepared media was then added. Next, a dialysis membrane (MWCO 12-14000 Da) containing 5 mL of fresh media was placed in each tube. Tubes were capped and placed in a shaking water bath. Solubility assessments were conducted after a 48 hour dwell period with the exception of fenofibrate, which was assessed after 96 hours. For assessment, tubes were taken from the water bath,

the dialysis membrane was removed, and its contents were extracted. One mL of media from the within the membrane was combined with 2 mL of methanol and vortexed for 5 seconds. The mixture was centrifuged at 8000 rpm and 4°C for 15 minutes. The resulting supernatant was filtered through 0.45 µm regenerated cellulose filters (Cronus) and diluted in mobile phase prior to analysis. Solubility values were quantified using HPLC-UV under the conditions specified in Table III. Calibrations curves with five standard concentrations were constructed for each test media. Standards were created by dilution of a stock solution, as described above, with fresh media using volumetric glassware. Solubility assessments were conducted in triplicate.

One-way analysis of variance (ANOVA) with a post-hoc Tukey's test was applied to identify statistically significant differences in solubility between various age-specific media (i.e. neonate-infant-adult). All statistical analyses was conducted using R statistical software (v 3.1.2). The investigation utilized a significance level of $p \leq 0.05$. Average solubility differences between developed pediatric media and the corresponding reference adult media were expressed as a ratio % ($\mu_{\text{pediatric}} / \mu_{\text{adult}} \times 100$). Values greater than 100% indicate compound solubility within the pediatric media exceeded the solubility observed in adults, whereas values less than 100% conveyed the opposite. To denote relevant discrepancies in solubility, reference points corresponding to ratios of 80% and 125% were used. These values parallel the 80-125% bioequivalence criterion as specified by the US-Food and Drug Administration (US-FDA) (16). Within the analysis, statistically significant mean ratios falling outside the pre-specified boundary range were estimated to be at an increased risk for exhibiting alterations in oral drug performance between children and adults. In contrast, when mean ratios were within the 80-125%, boundary, age-specific solubility differences were not expected to alter oral drug performance.

The influence of bile salts (NaTc) on modulating compound solubility within the developed biorelevant media was approximated using the equations presented by Mithani et al. (17),

$$\log SR = 2.09 + (0.64 \cdot \log P) \quad \text{Eq.1}$$

$$SC_{bs} = SR \cdot SC_{aq} \quad \text{Eq.2}$$

$$C_{sx} = C_{so} + (SC_{bs}) \cdot (MW) \cdot ([NaTc]) \quad \text{Eq.3}$$

where SR is the solubilization ratio, logP is the logarithm of the octanol-water partition coefficient, SC_{bs} is the bile salt solubilization capacity, SC_{aq} is the solubilization capacity of water, C_{sx} is the estimated compound solubility (mcg/mL) in the presence bile salts, C_{so} is the aqueous solubility (mcg/mL), MW is the compound specific molecular weight and [NaTc] is the media concentration (mM) of sodium taurocholate (bile salt). The equations, which describe the quantitative relationship between bile acids and compound solubility within aqueous based systems, incorporated bile salt concentrations for each age-specific media formulated with NaTc (FaSSGF, FaSSIF and FeSSIF). For neutral compounds (griseofulvin, spironolactone, carbamazepine and fenofibrate), experimentally determined aqueous solubility values served as inputs. For ionizable compounds (phenytoin-acid, indomethacin-acid, dapsone-base), pH specific aqueous solubility values, as estimated by the Henderson-Hassalbach equation, were utilized. The ratio of compound solubility, relative to adults, was estimated for each of the developed pediatric media (i.e. $pediatric_{pred}/adult_{pred} \times 100$). A comparison between these predictions, which solely account for the effect of bile acids, and measured values, which account of the influence of all media components, was instituted using the root mean square error (RMSE).

$$RMSE = \sqrt{\frac{1}{n} \sum_{j=1}^n \left(\left(\frac{\mu_{pediatric\ solubility(measured)}}{\mu_{adult\ solubility(measured)}} \times 100 \right) - \left(\frac{pediatric\ solubility(predicted)}{adult\ solubility(predicted)} \times 100 \right) \right)^2}$$

Eq.4

Here, the RMSE provides a quantitative assessment of the influence of media bile salts on modulating compound solubility. For example, high agreement between predicted and measured solubility ratios, as indicated by lower RMSE values, infers NaTc is primarily responsible for observed solubility changes.

Results:

Literature data utilized to define age-specific GI parameters were primarily sequestered from studies examining healthy/normal children in order to mitigate the confounding effects of altered health statuses. For example, several investigations examining fasting gastric pH in children focused on pre-operative subjects with no known GI disease undergoing elective surgery. However, due the scarcity of pediatric data, some investigations examining critically ill subjects (i.e. NICU, PICU, or ICU patients) as well as preterm neonates were included in the analysis. Such studies were additionally scrutinized to ensure their appropriateness towards defining GI parameters reflective of normal children. Assessments of gastric pH including critically ill subjects were restricted to studies where acid reducing agents (i.e. H₂ antagonists) were withheld (18-20). Two pediatric studies assessing fasting gastric pepsin levels included subjects deemed as critically ill (21, 22). As pepsin concentrations were presented as a percentage of adult values, data from these studies were compared to reference data (23) derived from critically ill adult subjects to normalize for any potential effects of illness. Similar to term neonates, preterm neonates by a gestational age of 34 weeks are expected to possess the ability to suckle and swallow to facilitate oral nutrition (24). Consequently, to minimize the effects of immaturity within the analysis, studies were delimited to those where the average postmenstrual age (gestational age + postnatal age) of subjects was approximately \geq 34 weeks.

Pediatric Fasted-State Simulated Gastric Fluid (P-FaSSGF):

Studies depicting gastric pepsin concentrations in pediatric subjects are presented as a percentage of adult values in Figure 1a. Reported concentrations were measured in a fasting state with or without histalog stimulation. Values derived from histalog stimulation tests were compared to adult subjects referenced within the same study (25). For other studies, reference adult values were ascertained from separate investigations by the same research group or investigations utilizing a similar assay technique. A segmented analysis towards neonatal subjects was only conducted in a single study (25). The investigation showed gastric pepsin concentrations approached infantile (1m-12m) levels after the first week of postnatal life. For example, neonates between 1-8 days postnatal age exhibited mean pepsin concentrations of ~15% of adult values while older neonates (10-32 days) and infants (67-110 days) both expressed similar mean concentrations of ~41% of adult values. Neonatal FaSSGF was developed based on the youngest cohort of subjects (i.e. those within the 1st week of life) to depict a state where the effects of development are most pronounced. Infant FaSSGF was formulated using pepsin concentrations summarized over several investigations. Concentrations of 15% and 25% of adult reference values (FaSSGF) were utilized for neonatal and infant FaSSGF, respectively.

Investigations depicting fasting gastric pH values in pediatric subjects are summarized in Figure 1b. Adult values represented by the mean from separate investigations, as summarized by Di Maio and Carrier (26), are displayed for reference. After the first day of life, fasting gastric pH rapidly normalizes towards adult values. Correspondingly, pediatric media (neonate and infant) representative of the fasted gastric state maintained the same pH as denoted by the reference adult media (i.e. FaSSGF - pH = 1.6).

A single pediatric study was identified that investigated fasting gastric osmolality in 40 postoperative infants with a mean age of approximately 8 months (27). The investigation depicted an average osmolality of 253 mOsm/L, which is more than twice the value of adult FaSSGF (120 mOsm/L). However, these findings may not be entirely reflective of healthy infants. In postoperative subjects,

administered medications and patient induced stress during surgery can effect gastric secretions and, thus, osmolality. Consequently, owing to the lack of appropriate data to establish a relationship between age and fasting gastric osmolality, the pre-established value from adults was employed to develop pediatric media.

Literature-based assessments of gastric bile acids and phospholipids (i.e. lecithin) in the fasting state were not available for pediatric subjects. As the gastric mucosa does not contain the capacity to produce or excrete bile, the presence of gastric bile acids are primarily the result of duodenogastric reflux, a normal physiological phenomenon documented in adults (28, 29). Therefore, it was postulated that intestinal bile levels would influence the magnitude of bile acids present within gastric fluids. With frequent feeding schedules, neonates and infants are often maintained within the fed-state during waking hours. As such, bile acid (i.e. NaTc) values within pediatric FaSSGF were derived using fed-state intestinal bile levels. The following formula was used to quantify NaTc concentrations in pediatric FaSSGF,

$$pFaSSGF_{[NaTc]} (uM) = \frac{pFeSSIF_{[NaTc]}}{FeSSIF_{[NaTc]}} \cdot FaSSGF_{[NaTc]} \quad \text{Eq.5}$$

where $pFaSSGF_{[NaTc]}$ is the bile acid (NaTc) concentration in pediatric FaSSGF, $pFeSSIF_{[NaTc]}$ is the NaTc concentration in pediatric FeSSIF, $FeSSIF_{[NaTc]}$ is the NaTc concentration in the reference adult FeSSIF media (10 mM), and $FaSSGF_{[NaTc]}$ is the NaTc concentration in reference adult FaSSGF media (80 uM). Bile acid values within pediatric FeSSIF media are presented in a forthcoming section. For lecithin, pediatric FaSSGF was formulated to maintain the same ratio of $[NaTc]/[lecithin]$ as depicted by adult FaSSGF. Compositions of the developed neonatal and infant FaSSGF media are presented in Table IV.

Pediatric Fed-State Simulated Gastric Fluid (P-FeSSGF):

The composition of FeSSGF is largely influenced by added meal components. In adult FeSSGF, cow's milk is typically incorporated as it contains similar ratios of carbohydrate/protein/fat as a typical

breakfast meal and avoids logistic difficulties associated with the use of homogenized solid meals (30, 31). To institute the most physiologically relevant depiction of gastric contents in children, pediatric media were formulated using two types of commonly marketed infant formula: Cow & Gate First Infant Milk (cow's milk-based formula) and Infasoy (soya-based formula). Development of separate pediatric FeSSGF media comprised of different formulas permitted for forthcoming solubility assessments to investigate the influence of pediatric diet on biorelevant solubility.

Gastric pH within the fed-state is dependent of several factors including feed composition and time of measurement (32, 33). Since many pediatric investigations administer various feeds (i.e. breast milk, infant formula, or D5W) and measure postprandial pH at selective time intervals, defining age-specific pH values was quite challenging. Adult FeSSGF represents a snapshot of the 'middle' phase of gastric digestion between 75 and 165 minutes post-meal ingestion (2). The pH of adult FeSSGF was derived from Kalantzi et al.'s study, where a liquid meal consisting of 500mL Ensure plus® was administered to 20 healthy subjects (33). The study denoted a pH of 5 as the approximate average over the abovementioned time period. However, in a separate investigation by Dressman et al. (34), where gastric pH was monitored following ingestion of a standard solid meal (1000 Kcal), postprandial pH values differed from the results attained by Kalantzi et al. Following administration of a solid meal, gastric pH decreased towards fasting values at a faster rate compared to subjects administered a liquid meal. For example, median pH values persisted above 3 for approximately 60 minutes vs. > 180 minutes following solid meal vs. liquid meal ingestion, respectively (33, 34). As solid foods are anecdotally the most common form of meals consumed by adults, comparison of postprandial pH changes between children, administered a typical meal (i.e. formula), and adults, administered a solid meal, were used to define pH for the developed pediatric FeSSGF. Sondheimer et al. investigated the influence of postnatal age (PNA) on gastric pH in healthy preterm neonates (35). In-situ pH monitoring was conducted following administration of infant formula in two groups of neonates aged 2-6 days and 7-15 days PNA. Comparing pH values at approximately 120

minutes post-meal (i.e, mid-point of the 75-165 minute time frame) between the cohort of older preterm neonates and adults, as reported by Dressman et al., pH was found to be higher (0.7-1.8 units) among neonates. As a result, neonatal FeSSGF was formulated to adopt a slightly higher pH (pH = 5.7) as compared to the reference adult FeSSGF (pH = 5).

Osmolality of pediatric FeSSGF was defined by two investigations. The first, conducted by Billeaud et al. (36), characterized gastric osmolality among 15 low birth weight neonates with a mean PNA and gestational age (GA) of 8 days and 35.4 weeks, respectively. Eight test feeds, each differing in osmolality, were administered. The study noted a positive linear relationship between feed and gastric osmolality over the 3 hour study period. In a separate investigation by Thatrimontrichai and Janjindamai (37), three separate expressed breast milk feeds, which ranged in osmolality due to the addition of mineral/vitamin supplements, were tested in 26 neonate/infant subjects with a median PNA and GA of 30 days and 30 weeks, respectively. Within the study, meals with higher osmolalities were found to be associated with comparatively higher gastric osmolalities over the 1 hour test period. A linear regression model depicting the degree of association between feed osmolality and 60 minute postprandial gastric osmolality was developed based on the results of Billeaud et al.'s (36) investigation (Figure 2). Although a 60 minute sampling point was not obtained during the original study, the value was estimated as the average between the 45 and 90 minute sampling intervals. The validity of the derived regression equation was tested by comparing gastric osmolality predictions to the data presented within Thatrimontrichai and Janjindamai's study (37). The results of this comparison are depicted in Table V. Estimates from the regression equation were within 8% of measured values. As such, the equation was deemed appropriate for defining osmolality in neonatal FeSSGF. Although a sampling point of 60 minutes was clearly outside the time frame used to define adult FeSSGF (75-165 minutes), children, especially those within the youngest age groups, are typically fed on a more consistent basis during waking hours (i.e. every 2-3 hours). Correspondingly, defining gastric osmolality in children based on one hour postprandial values

may provide an age appropriate representation of the ‘middle’ phase of gastric digestion, which adult FeSSGF is formulated to mimic.

Since basal gastric volumes in infants are minute (38), the composition of gastric fluids postprandially can likely be attributed to the properties of the ingested meal. As such, the buffering capacity of pediatric FeSSGF was determined based on the buffering capacity of infant formula incorporated into the media. Since two separate neonatal media, one based on cow’s milk formula and the other based on soy formula, were developed, buffering capacity determinations for each respective formula were required. Determinations were conducted at pH 5.7, the desired pH of neonatal FeSSGF. The buffering capacity (mean \pm SD) of cow’s milk formula at pH 5.7 was 14.03 ± 0.164 . Soy-formula at pH 5.7 displayed similar a buffering capacity (14.94 ± 0.318 mEq/L/ Δ pH). For simplicity, neonatal FeSSGF based on cow’s milk formula and soy formula media were prepared to target a buffering capacity 15 mEq/L/ Δ pH. Compositions of the developed P-FeSSGF media are depicted in Table VI.

Appropriate information to define infantile fed-state gastric fluids (i.e. 1-12m) was not attained from the literature. As a result, an infant FeSSGF media was not developed. However, as the composition of FeSSGF is primarily attributed the contents of the added meal component, assessments conducted in neonatal media which incorporate infant formula should provide a general indication of expected solubility changes in infants consuming similar feeds. In addition, comparisons between neonatal media that are similar in all respects with the exception of the type of meal component added (i.e. cow’s milk-based formula vs. soy-based formula) provide an assessment of the impact of feed composition on biorelevant solubility.

Pediatric Fasted-State Simulated Intestinal Fluid (P-FaSSIF):

Intestinal pH values depicted in the literature are summarized as a function of postnatal age in Figure 3a. The majority of pediatric data was attained from the distal duodenum though a few studies that sampled from the proximal jejunum were also included. Adult pH is depicted as mean values from separate investigations, as summarized by Fuchs and Dressman (39). Studies investigating intestinal pH in children, especially in the youngest age groups, were not widely published in the literature. In addition, data obtained from adults encompassed a large degree of variability. Consequently, no distinct relationship between age and fasted-state intestinal pH was observed. Pediatric media were subsequently formulated using the same pH as denoted for the adult reference media (i.e. FaSSIF – pH = 6.5)

Fasting bile salt concentrations from the proximal small intestine are depicted as a function of age in Figure 3b. A large degree of variability was apparent in both children and adults as denoted by the spread of data. Discernable differences between pediatric age groups (i.e. neonates, infants) and adults were not visually evident. Furthermore, the linear association between the logarithm of age and bile acid concentrations was negligible ($R^2=0.05$) among pediatrics. Due the substantial degree of variability between pediatric studies, P-FaSSIF was developed to assess two potential scenarios. In one media, bile salt concentrations were formulated to be 150% of adult values. In the second media, concentrations were formulated to be 50% lower than adults. A pediatric media where bile salt concentrations were similar to adult values did not necessitate development of a new media as this scenario was already depicted by the adult reference. Developed media represent hypothetical depictions of bile acid concentrations within a biologically plausible range. Correspondingly, the magnitude of compound specific solubility differences denoted in such media provides an indication of whether additional pediatric investigations are required to define bile acids within the fasted-state intestine.

No pediatric data pertaining to phospholipids (i.e. lecithin), buffering capacity, and osmolality of intestinal fluids in the fasted-state were ascertained. Pediatric media were therefore formulated to

maintain the same [NaTc]/[lecithin] ratio as depicted in the adult reference media (FaSSIF). Buffering capacity and osmolality were also defined using adult values. Compositions of the proposed P-FaSSIF media are presented in Table VII.

Pediatric Fed-State Simulated Intestinal Fluid (P-FeSSIF):

Fed-state duodenal pH values from separate pediatric and adult investigations are presented in Figure 4a. Of the few studies presented amongst pediatrics, pH values appear to overlap with those depicted from adults. Owing to the disparate nature of available data, pH differences between each age group (neonate, infants, and adult) could not be fully elucidated. Pediatric FeSSIF media were therefore formulated using the same pH as the adult reference (i.e. pH = 5.8).

Assessments of postprandial intestinal osmolality amongst pediatrics were also scarcely published within the literature. A single study conducted by Billeaud et al. (36), which was also utilized to define pediatric FeSSGF osmolality, was identified. Duodenal osmolality was assessed in 15 low birth weight neonates following administration of a variety of feeds, each varying in osmolality. A positive linear association between feed and duodenal osmolality was found. A regression model was constructed using a congruent approach as previously discussed for defining FeSSGF osmolality (Figure 4b). Although a suitable coefficient of determination ($R^2 = 0.92$) between feed osmolality and 60 minute postprandial duodenal osmolality was attained, a second study from which the model could be evaluated within pediatrics was unavailable. As an alternative assessment, the model was utilized to estimate to duodenal osmolality in two adult studies. Mean duodenal osmolality values of approximately 405 and 392 mOsm/kg were observed 1 hour following administration of liquid meals containing 610 and 670 mOsm/kg in separate investigations conducted by Kalantzi et al. (33) and Clarysse et al. (40), respectively. The proposed regression model provided duodenal osmolality estimates of 430 (6% over-prediction) and 454 (16% over-prediction) mOsm/kg for each respective adult investigation. Although derived from a cohort

of neonatal subjects, the model exhibited an adequate predictive capacity in adults. By extension, its use for estimating intestinal osmolality amongst pediatrics (neonates and infants) was considered to be appropriate. The osmolality of neonatal FeSSIF was formulated to reflect two separate feed types, breast milk, with a reported osmolality of ~300 mOsm/kg (36, 41), and cow's milk-based formula with a measured osmolality of 368 mOsm/kg (Cow & Gate First Infant Milk). For FeSSIF reflective of older children (i.e. infants) where weaning is commonly instituted, only a single feed type was investigated, cow's milk formula. Using the aforementioned regression equation, osmolality of the developed pediatric FeSSIF was defined as 300 and 330 mOsm/kg post-administration of breast milk and cow's milk-based formula, respectively.

Fed-state duodenal bile salt concentrations among pediatrics and adults are summarized in Figure 4c. A positive linear association between the logarithm of age and duodenal bile acid concentrations was denoted among children ($R^2 = 0.54$). Bile acid concentrations among adults displayed variability, but for the most part studies depicted a mean value of approximately 10 mM, corresponding to the concentration of the reference adult media (FeSSIF v2). Mean bile acid concentrations among neonates (0-28 days) and infants (1-12m) were approximately 25% (i.e. 2.5 mM) and 75% (i.e. 7.5 mM) of adult values, respectively. Pediatric FeSSIF were subsequently formulated using these bile acid concentrations.

Pediatric studies characterizing concentrations of fat digestion products in the intestinal lumen have not been reported in the literature. However, since the quantity of such products is dependent on the interrelationship between fat digestion and absorption, an examination of these processes was instituted in order to derive age-dependent estimates. In newborns, concentrations of pancreatic colipase-dependent triglyceride lipase, the enzyme primarily responsible for lipid metabolism in adults, is decreased (42). Despite this, the presence of auxiliary enzymes such as human gastric lipase, pancreatic lipase-related protein 2 and bile salt-stimulated lipase, are postulated to provide an efficient means of

lipid digestion for newborns (42). In terms of absorption, breast-fed neonates exhibit fat absorption coefficients reminiscent to that of adults despite lower duodenal bile acid concentrations (43). It was therefore inferred that the developmental capacity of both fat digestion and absorption were comparable to adults amongst this pediatric cohort. FeSSIF media reflective of breast-fed neonates were correspondingly formulated using the same concentrations lipid digestion products (glyceryl monooleate and sodium oleate) as defined for the adult reference media (i.e. FeSSIF).

However, among formula-fed neonates, fat absorption coefficients are notably lower compared to their breast-fed counterparts as well as adults (43, 44). Unlike breast-fed neonates, intestinal bile concentrations in formula-fed neonates were found to exhibit a positive linear correlation with percent fat absorption (43, 44). To decipher whether a deficiency in lipid absorption or lipid digestion was the primary factor limiting internalization of fats in formula-fed neonates, pathophysiological information pertaining to necrotizing enterocolitis (NEC) was used. NEC is a debilitating inflammatory GI condition occurring typically in preterm neonates but also uncommonly in term neonates. In both groups, the incidence of NEC is substantially higher in formula-fed subjects as compared to those receiving enteral feeds with breast milk (45, 46). Though the mechanism of pathogenesis of NEC is not completely understood, one theory as described by the work published by Penn et al. (47) identified the presence of elevated concentrations of free fatty acid (FFA) as the culpable factor. The study found lipase digestion of formula, but not human milk, exhibited a cytotoxic effect in three different cell types. Furthermore, digested formula displayed significantly greater levels of FFA compared to lipase digested human milk. Based on this finding in conjunction the prevalence of NEC amongst the youngest cohort of neonates, it was inferred that the process of lipid digestion was not developmentally impaired in formula-fed neonates. Hence, the decreased capacity for fat internalization was attributed to an inadequate lipid absorptive capacity in such subjects. Correspondingly, formula-fed neonates would be expected to exhibit higher luminal concentrations of lipid digestion products. Using 75% as the average coefficient of fat

absorption in formula-fed neonates (42-44), the concentration of lipid digestion products (glyceryl monooleate and sodium oleate) was estimated to be 1.33x (i.e. $1/0.75$) greater in the intestinal lumen of neonates that are formula-fed compared those that are breast-milk fed. P-FeSSIF media pertaining to formula-fed neonates was developed based on the above assertion. In infants, where luminal bile acid concentrations are higher, fat absorption is not expected to exhibit developmental impairment. Pediatric FeSSIF reflective of formula fed infants was therefore formulated using the same concentrations of fat digestion products as depicted for the adult reference media.

No pediatric studies investigating buffering capacity and concentrations of phospholipids (i.e. lecithin) within the fed-state intestinal lumen were obtained. Buffering capacity of the developed pediatric media were consequently formulated using a value of 25 mEq/L/ Δ pH, the adult reference value. Using a similar approach as employed for P-FaSSGF and P-FaSSIF, lecithin concentrations were fixed to provide the same ratio of [NaTc]/[lecithin] as expressed by the reference adult media (i.e. FeSSIF.v2). The compositional details of developed P-FeSSIF media are depicted in Table VIII.

Solubility Assessments:

Solubility determinations for six of the seven compounds (carbamazepine, dapsone, griseofulvin, indomethacin, phenytoin and spironolactone) were conducted in age-specific media representative of all four gastrointestinal states: FaSSGF, FeSSGF, FaSSIF and FeSSIF (Figure 5). For fenofibrate, use of the predefined equilibrium dialysis method did not serve as a suitable technique for solubility determinations in fed-state gastric media. Penetration of fenofibrate through the dialysis membrane was inefficient during the selected study interval (96 hours) and, as a result, solubility could not be quantified. Figure 6

depicts solubility determinations of fenofibrate in age-specific media reflective of the remaining three gastrointestinal states: FaSSGF, FaSSIF and FeSSIF.

For pediatric media representative of the fasted gastric state (i.e. P-FaSSGF), three compounds (carbamazepine, indomethacin and fenofibrate) exhibited mean solubility values below the 80 to 125% reference range, relative to adults. Though for indomethacin, this difference was not statistically significant. Relative solubility changes depicted between neonatal and infant FaSSGF were consistent in terms of direction and magnitude for six of seven compounds. Only one compound, carbamazepine, displayed a statistically significant difference in solubility between neonatal and infant FaSSGF.

Solubility assessments in neonatal fed-state gastric media (i.e. P-FeSSGF), developed using cow's milk-based or soy-based formula, were compared to solubilities attained in adult FeSSGF formulated with cow's milk. Five compounds (carbamazepine, dapson, griseofulvin, phenytoin and indomethacin) exhibited changes in solubility that fell outside the aforementioned reference range in at least one of the developed neonatal media. A trend towards lower solubility values in neonatal media was found for four of the compounds (carbamazepine, dapson, griseofulvin and phenytoin). For indomethacin, a weak acid ($pK_a = 4.5$), an increase in solubility compared to adult media was observed that was attributed, in part, to the higher pH of neonatal FeSSGF. Statistically significant differences in solubility between neonatal media formulated using either cow's milk-based or soy-based formula was observed in 4/6 compounds. For carbamazepine, solubility values in media comprised with cow's milk formula was greater than that of media comprised with soy formula. For dapson, phenytoin and indomethacin the opposite was observed. In contrast, for spironolactone and griseofulvin, no statistically significant difference in compound solubility was noted between the respective neonatal media.

Since a consensus regarding differences in bile salt concentrations between children and adults within the fasted-state intestine was not achieved, compound solubility was investigated based on two

theoretical media that incorporated bile salt concentrations of 150% (4.5 mM) and 50% (1.5 mM) of those in adults. For the majority of compounds (6/7), solubility determinations in both media fell within an 80% to 125% range when compared to adult values. However, for fenofibrate ($\log P = 5.3$), solubility in P-FaSSIF-50% media was 56% of the value observed in the adult reference.

Solubility determinations conducted in pediatric media reflective of the fed-state intestine (i.e. P-FeSSIF) were compared to values attained in adult FeSSIF. For three of seven compounds (fenofibrate, griseofulvin and phenytoin), mean solubilities of less than 80% of adult values were observed in at least one of the formulated P-FeSSIF. These relevant solubility alterations were exclusively found in neonatal media. In comparison, mean solubility values in infant FeSSIF fell within 80-125% of adult values for all compounds investigated. A general trend towards statistically significant lower solubilities in neonatal media compared to infant FeSSIF was observed in five of seven compounds. Statistically significant solubility differences between neonatal media formulated to depict intestinal fluids following administration of cow's milk-based formula (Pnc-FeSSIF) or breast milk (Pnb-FeSSIF) were denoted for three compounds (griseofulvin, spironolactone, and phenytoin). A higher solubility was observed for griseofulvin in Pnc-FeSSIF though in both neonatal media, values were below the 80-125% reference range. Solubility was also greater in Pnc-FeSSIF for spironolactone but, in this case, solubility values in both media fell within the 80-125% reference range. In contrast, for phenytoin, a higher solubility was observed in Pnb-FeSSIF. Solubility in Pnb-FeSSIF fell within 80-125% of adult values, but for media depicting formula-fed neonates (Pnc-FeSSIF), the mean solubility was well below the 80% reference point.

Changes in compound solubility between pediatric and adult media induced by alterations in bile salt concentrations were estimated according to the equations proposed by Mithani et al (17). These values are displayed in Figures 5 and 6 (red dots) for media formulated with NaTc (i.e. FaSSGF, FaSSIF, and FeSSIF). Table IX displays RMSE values between predicted and measured solubility ratios for the

developed pediatric media in order of increasing compound lipophilicity. For the two least lipophilic compounds investigated (dapson and griseofulvin), predictions made using Mithani et al.'s equations were within a RMSE of 10%. In these cases, the equations provide an acceptable approximation of the direction and magnitude of solubility changes observed in pediatric media. As compound lipophilicity increased, a departure between predicted and measured solubility ratios was observed as indicated by larger RMSE values. For such compounds, the predicted magnitude of solubility changes due to alterations in media NaTc content were typically overstated when compared to measured values.

Discussion:

Based on an assessment of the current literature, biorelevant media simulating the unique intricacies of the upper gastrointestinal tract (stomach and proximal small intestine) in pediatrics were developed and utilized to estimate compound specific solubility. Preferably, solubility comparisons between pediatrics and adults should be conducted using ex-vivo luminal fluid samples, but due to the numerous logistical and ethical constraints associated with obtaining of such samples in pediatrics, the use of biorelevant media was deemed as a suitable approach. In adults the appropriateness of biorelevant media has been established by investigations depicting strong positive correlations in compound solubility between simulated and human intestinal fluids (48, 49).

Pediatric fed-state gastric media (P-FeSSGF) was formulated using either cow's milk-based or soy-based formula to assess the impact of feed type on compound solubility. The use of human breast milk within the investigation was precluded due to logistic issues associated sample obtainment and uniformity. In terms of uniformity, the composition human breast milk is well known to exhibit both intra- and inter-subject variability in composition (50). Infant formulas, however, are subject to quality control inspections to ensure batch-to-batch uniformity, ensuring biorelevant media are prepared in a reproducible fashion. Marketed infant formulas are designed to mimic the composition of human breast

milk with regards to the proportions of energy provided from protein, fats, and carbohydrates (51). In human breast milk, proteins are predominantly comprised of two forms, whey and casein, in a ratio of 60:40, respectively. In contrast, the whey-to-casein ratio of cow's milk is 18:82 (51). To address this discrepancy, many formulas, including Cow & Gate First Infant Milk (cow's milk based), have introduced additional amounts of whey protein in order to mimic ratios observed in human milk (51). The influence of casein on compound solubility has previously been depicted for the anticoagulant dicumarol, where increases in casein concentration corresponded to higher dicumarol solubility values (52). Despite supplementation with vitamins and minerals, infant formula cannot fully reproduce the biological complexity of human breast milk which contains antibodies, enzymes, and growth factors (51). However, cow's milk formula does provide a suitable approximation in terms of macronutrient composition and protein type that is free from the inherent variability associated with breast milk. As such, solubility studies conducted in neonatal FeSSGF comprised with cow's milk formula may encompass applicability towards breast fed neonates.

Concentrations of media components were formulated to represent the average tendency over a specific age range. To summarize age-specific data from the literature, the investigation utilized non-weighted arithmetic means. Though simplistic in nature, use of the arithmetic mean was preferred over other more robust computational or statistical analyses based on several considerations. First, there is the relative disparity of literature investigations devoted to defining the composition of luminal fluids in pediatrics compared to adults. For many media components only a handful of studies were available to quantify differences between subsequent age groups. Of studies obtained, high degrees of variability were typically noted. This is likely attributed to the dynamic nature of the developmental process, where the composition of luminal fluids continually change as children mature. Due to this disparity of available data and its inherent variability, employment of statistical tests to identify significant differences in component concentrations between adjacent age groups were not applicable. Similarly, the use of

581 regression analyses were typically unable to establish meaningful correlations between parameter values
582 and age. A second consideration for the preferential use of non-weighted arithmetic means was due to
583 the precarious nature of qualifying investigations. A large majority of pediatric studies were completed
584 over three decades ago, where differences in reporting standards and quantitative techniques were wide-
585 ranging. Employment of a non-weighted approach was instituted to simplify the analysis though,
586 understandably, the method lacks the informative capacity of approaches that consider study quality, as
587 frequently adopted by systematic reviews (53). Finally, studies varied in terms of data presentation,
588 making implementation of weighted averages difficult. Reporting of variability associated with luminal
589 fluids components was inconsistent between investigations. For example, separate studies utilized a
590 variety of measures including standard deviation, range, or interquartile range. Additionally, the number
591 of subjects allocated to specific age ranges were not identified by some investigators (21). Due to this lack
592 of consistency between studies, employment of a weighted mean was precluded in favor of a non-
593 weighted approach. As the arithmetic mean does not provide an indication of parameter variability, the
594 analysis is unable to depict expected variations within the population. However, as biorelevant media is
595 developed to represent luminal fluids in an average individual, descriptions of parameter variability were
596 unnecessary.

597 Due to the disparate nature of available pediatric data, the quantitative value of many media
598 components were based on biological inferences or adoption of adult values. For example pediatric
599 investigations pertaining to luminal concentrations of pepsin, phospholipids, fat digestion products, and
600 osmolality were either scarcely reported or lacking within the literature. The proposed age-specific media
601 attempted to approximate the *in vivo* composition of pediatric luminal fluids based on a current state of
602 knowledge. As future investigations are obtained, such formulations should undoubtedly be modified to
603 provide greater degrees of biological relevance.

In addition to its primary goal of facilitating suitable IVIVC, biorelevant media should demonstrate a practical degree of stability. Apart from noticeable changes in visual appearance, media stability can be formally evaluated by assessing for alterations in physicochemical parameters under ambient and test conditions. In Jantratid et al.'s original publication (2), which described the reference adult media utilized by this investigation, stability was evaluated through measurements of media pH, buffering capacity and osmolality. For adult FeSSGF and FeSSIF-v2, consistency in physicochemical parameters were observed under ambient conditions over a 72 hour study period. In addition, with the exception of minor changes in osmolality, the abovementioned media demonstrated stability under test conditions of 37°C over the same time period. Changes in media physicochemical properties (i.e. poor stability) during solubility assessments may lead to corresponding changes in compound specific saturation solubility. In the current investigation, solubility assessments in fed-state simulated gastric media comprised of cow's milk (FeSSGF) and infant formula (neonatal FeSSGF) were conducted at 37°C after 48 hours for most compounds. Though stability studies were not conducted within the developed pediatric FeSSGF, it was inferred that stability would be similar to that of adult FeSSGF. However, if large scale implementation of the depicted pediatric media is desired, future research evaluating media stability will certainly be required.

For the majority of study compounds, solubility assessments proceeded without issue. Though for fenofibrate, the most lipophilic compound evaluated ($\log P = 5.3$), logistic issues materialized with solubility determinations in biorelevant media reflective of the fed gastric state. For FeSSGF media, the study employed equilibrium dialysis to assess compound solubility. Though this technique proved effective for most compounds, it was unsuitable for fenofibrate. Following a 96 hour dwell period, fenofibrate concentrations within the membrane were below the limit of quantification. This result may indicate inadequate permeation of the dialysis membrane by fenofibrate in milk or formula-based samples. A congruent example is demonstrated by the *in vivo* pharmacokinetics of fenofibrate. In humans,

fenofibrate exhibits extensive protein binding (~99%) and, as such, filtration by hemodialysis is not considered effective (54, 55). Based on this assessment, use of equilibrium dialysis was not considered feasible for determination of fenofibrate solubility in fed state gastric media. These values were correspondingly excluded from the analysis.

Solubility assessments were confined to BCS Class II compounds, where limitations in absorption are primarily attributed to inadequate drug solubility. For such compounds, differences in luminal solubility may signify alterations in oral drug performance (56). To maintain a degree of biological relevance, the analysis was further limited to compounds where documented or investigational uses in both children and adults have been depicted. To identify relevant changes in the age-specific solubility, the study utilized the same threshold as depicted by the US-FDA for attainment of *in vivo* bioequivalence (i.e. 80-125%) (16). It should be noted, however, that solubility is only one parameter which can exert an effect on oral compound absorption. Other parameters including gastric emptying time, small intestinal transit time, intestinal permeability, gut metabolism, luminal degradation and presence of intestinal transporters may also impart an influence *in vivo*. In order to fully elucidate the impacts of growth and development on oral compound absorption, a more comprehensive analyses such as physiologically-based pharmacokinetic (PBPK) modeling would be required to integrate age-dependencies in all the aforementioned parameters. The presented analysis which focusses on biorelevant solubility as a surrogate for oral compound performance was therefore an overt simplification. However, this approach was justified based on the cohort of compounds assessed, which was confined to solubility-limited (BCS Class II) drugs.

Compared to adult media, solubility in pediatric fasted-state gastric media (i.e. P-FaSSGF) was both statistically different ($p \leq 0.05$) and outside the purported bioequivalence criterion for two compounds, fenofibrate and carbamazepine. For fenofibrate, mean solubility values in adult FaSSGF and pure water

were comparable at 0.281 and 0.206 mcg/mL, respectively. In contrast, solubility within adult fasted-state intestinal media (i.e. FaSSIF) was considerably greater (2.42 mcg/mL). The discrepancy in solubility values between FaSSGF and FaSSIF provides an indication of the relative influence of each state on modulating oral absorption. In this case, due to its poor solubility in comparison to intestinal fluids, fasted state gastric fluids are unlikely to play an influential role on modulating the extent of fenofibrate absorption. Solubility alterations observed in P-FaSSGF were therefore not postulated to impact the oral performance of fenofibrate in children. Differences in solubility between neonatal and infant FaSSGF reached a statistically significant threshold for only one compound, carbamazepine. Though for both media, the mean solubility fell outside the bioequivalence threshold when compared to adult values. Based on this analysis, an argument may be formed as to the need for separate pediatric media since solubilities in neonate and infant FaSSGF appear to be similar in most cases. The current investigation focused on solubility, a compound specific property. However, in terms of establishing IVIVC for solid dosage forms, biorelevant media is typically employed within dissolution tests to assess formulation properties (57). In addition to modulating solubility, media components that are age-specific may also exert an influence on the rate of compound release and subsequent dissolution from a formulation. For example, the addition of pepsin into biorelevant media has been demonstrated to decrease surface tension (3). For specific formulations, such changes can exert of an effect on the rate of compound dissolution (58). Also, the presence of pepsin within dissolution media can facilitate effective compound release from cross-linked gelatin capsules (59). Therefore, although comparable solubilities were observed for neonatal and infant FaSSGF media, use of separate age-specific media may be justified for use in dissolution testing.

Solubility assessments in age-specific FaSSGF media were conducted for six compounds. For the majority of compounds (5/6), the mean solubility in neonatal media, comprised of either cow's milk-based or soy-based formula, fell outside the 80-125% bioequivalence criterion in relation to adult media comprised of milk. In addition, statistically significant differences in compound solubility between

pediatric media comprised with cow's milk-based and soy-based formula were observed in four compounds. These results infer that differences in feed composition between children as well as between children and adults can impart relevant changes in gastric solubility and, potentially, affect oral compound performance.

Of the limited pediatric investigations examining luminal fluids within the fasted-state proximal intestine, bile salt concentrations were found to exhibit a high degree of variability without any apparent age dependency. To explore the impact of such variations, two FaSSIF media were developed with bile salt concentrations of 50% (1.5 mM) and 150% (4.5 mM) of adult values. For the majority of compounds (6/7), mean solubility values within the two proposed P-FaSSIF media fell within an 80-125% range from adult values. However for the most lipophilic compound, fenofibrate ($\log P = 5.3$), solubility in P-FaSSIF media containing 1.5 mM NaTc was 56% of adult values. If such a media is reflective of *in vivo* luminal fluids in children, the observed change in solubility may signify an alteration in fenofibrate oral performance compared to adults. Prospectively, hydrophobic compounds are expected to play an increasingly important role in therapeutics as use of drug discovery techniques such as high-throughput screening typically produces candidate compounds of higher lipophilicity (60). To provide an accurate depiction of luminal solubility for such compounds, a consensus regarding intestinal bile salt concentrations in pediatrics is needed. This demonstrates a need for more high quality studies characterizing gastrointestinal physiology in pediatrics.

Solubility assessments conducted in fed-state intestinal media representative of infants were within 80-125% of adult values for all 7 compounds tested. Such a result was unsurprising as infant and adult media were compositionally similar, aside from small deviations in bile salt content, lecithin, and osmolality. Two neonatal media were formulated to reflect differences in intestinal fluid composition following administration of breast milk or cow's milk-based formula. Mean compound solubility values in

neonatal media fell outside the 80-125% criterion from adult values for 3 of the 7 compounds examined. Statistically significant differences in solubility between media reflective of breast-fed and formula-fed neonates was observed for 3 compounds. The relative magnitude of these differences appeared to be compound specific. For example, spironolactone solubility in intestinal media reflective of breast and formula fed neonates were 83% vs. 91% of adult values, respectively. In contrast, for phenytoin a larger discrepancy between solubility ratios was observed (92% vs. 61% of adult values, respectively). These findings demonstrate the potential impact of different feed types on intestinal compound solubility.

The study also included an evaluation the relative importance of bile salts in modulating compound solubility within the developed pediatric media. Predictive equations presented by Mithani et al. (17) were used to estimate the impact of alterations in bile salt content on compound solubility. Measured solubility values, which are influenced by all media components, were compared to estimated values, which only account for differences in media bile salts, using RMSE. The analysis demonstrated a decreased predictive capacity of the aforementioned equations (ie. larger RMSE values) as compound lipophilicity (logP) increased. This indicates that as compound lipophilicity increases, other media components, aside from bile salts, exert a more pronounced role in modulating compound solubility. For example, the capacity of media components such as buffer (sodium phosphate), fat digestion products (sodium oleate) and salt (sodium chloride) to modify compound solubility has previously been demonstrated within the literature (10).

Conclusion:

The current investigation strove to appropriately depict the *in vivo* composition pediatric luminal fluids based on the current literature and represents an initial foray into the development of pediatric biorelevant media. To increase the biological applicability of future iterations of such media, it is clear prospective studies focused on defining the composition of the pediatric lumen under varying conditions

is required. For 6 of the 7 BCS Class II compounds investigated, solubility fell outside an 80-125% range from adult values in at least one of the developed pediatric media. This result demonstrates the impact of age-related alterations in GI fluid composition on compound solubility. Solubility represents an integral component of the BCS, a framework which is extensively utilized by both industry and regulatory bodies to guide drug development in adults. The utility of a similar classification system in pediatrics is in part contingent on our understanding of how developmental differences between children and adults translates to alterations definable properties such compound solubility. The investigation sought to address this concern and, in turn, provides a dialogue surrounding the future development of a pediatric-focused BCS.

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1020

1021 Table I: Composition of adult biorelevant media

Component	FaSSGF	FeSSGF	FaSSIF.v2	FeSSIF.v2
Sodium Taurocholate	80 (uM)	-	3 (mM)	10 (mM)
Lecithin	20 (uM)	-	0.2 (mM)	2 (mM)
Pepsin (mg/mL)	0.1	-	-	-
Sodium Chloride (mM)	34.2	237.02	68.62	125.5
Acetic Acid (mM)	-	17.12	-	-
Sodium Acetate (mM)	-	29.75	-	-
Maleic Acid (mM)	-	-	19.12	55.02
Sodium Hydroxide (mM)	-	-	34.8	81.65
Glyceryl Monooleate (mM)	-	-	-	5
Sodium Oleate (mM)	-	-	-	0.8
Milk:Buffer	-	1:1		-
HCl/NaOH qs	pH 1.6	pH 5	pH 6.5	pH 5.8
pH	1.6	5	6.5	5.8
Osmolarity (mOsm/kg)	120.7	400	180	390
Buffering Capacity (mEq/L/ ΔpH)	-	25	10	25

1023 *adult media compositions as described in Jantratid et al. (2)

1024

1025 Table II: Compound physicochemical properties

Compound	Molecular Weight (g/mol)	LogP	pKa (acid/base)
Dapsone	248	0.97	2.4 (base)
Griseofulvin	353	2.18	- 1027
Carbamazepine	236	2.45	-
Phenytoin	252	2.47	9.5 (acid)
Spirolactone	417	2.78	- 1028
Indomethacin	358	4.27	3.8 (acid)
Fenofibrate	361	5.3	- 1029

1030 *physicochemical data obtained from DrugBank (61)

1031

1032

1033 Table III: HPLC-UV analytic conditions

Column	Compound	Mobile	Q ^a (ml/ min)	Temp (°C)	Inj Vol (μL)	λ ^b (nm)	R _t ^c (min)	Reference
1	Carbamazepine	MeOH/ Water (60:40)	1	20	50	285	6.6	(62)
2	Dapsone	Water with ammonium acetate 0.0286M / MeOH (70:30)	1	20	10	295	5.6	(63)
2	Fenofibrate	MeOH/ Acetate buffer 0.010M pH=3.7 (82:18)	1	25	80	286	6.5	(64)
2	Griseofulvin	MeOH/ Water (65:35)	1	20	20	292	4.5	(65)
2	Indomethacin	MeOH/ Water with 1.67% orthophosphoric acid (70:30)	1	23	100	270	9.9	(66)
2	Phenytoin*	Water/ AcN (50:50)	0.5	20	10	210	5.6	(67)
2	Spirolactone	MeOH/ Water (70:30)	1	20	40	237	5.7	(68)

1034

1035 Column 1: Hypersil (Thermo) BDS -C18 250 x 4.6mm - 5 μm

1036 Column 2: Zorbax SB-C18 150 x 4.6mm – 3.5μm

1037 a - Q = flow rate

1038 b - λ = UV wavelength

1039 c - R_t = retention time

1040 * - HPLC conditions altered for solubility assessments with FeSSGF media due to interference with media
 1041 components. Mobile phase (Water/AcN – 60:40), Q (1ml/min) and R_t=4.8 mins.

1042

1043

1044

1045 Table IV: Pediatric Fasted-State Simulated Gastric Fluids (P-FaSSGF)

Component	Pn-FaSSGF ^a	Pi-FaSSGF ^b
Sodium Taurocholate (uM)	20	60
Lecithin (uM)	5	15
Pepsin (mg/mL)	0.015	0.025
Sodium Chloride (mM)	34.2	34.2
HCl qs	pH 1.6	pH 1.6
pH	1.6	1.6
Osmolarity (mOsm/kg)	120.7	120.7
Buffering Capacity (mEq/L/ ΔpH)	-	-

- 1046
- 1047 (a) *Pn-FaSSGF – pediatric fasted-state gastric media representative of neonates (0-28 days)*
- 1048 (b) *Pi-FaSSGF – pediatric fasted-state gastric media representative of infants (1-12 months)*

1049

1050

1051 Table V: Predictive performance of the osmolality regression equation

Feed Osmolality (Median - mOsm/kg) ^a	Measured Gastric Osmolality - 60 min postprandial (Median - mOsm/kg) ^a	Predicted Gastric Osmolality – 60 min postprandial (mOsm/kg) ^b	% Prediction Error ((Pred– Obs) / Obs) x 100
344	354	327	-7.6 %
426	383	368	-3.9 %
315	315	313	-0.6 %

1052

1053 (a) Values derived from Thatrimontrichai and Janjindamai (37)

1054 (b) Predictions based on regression model, as derived from Billeaud et al. (36)

1055

1056

1057 Table VI: Pediatric Fed-State Simulated Gastric Fluids (P-FeSSGF)

Component	Pnc-FeSSGF ^a	Pns-FeSSGF ^b
Sodium Chloride (mM)	100.35	94.79
Acetic Acid (mM)	7.25	7.25
Sodium Acetate (mM)	64.65	64.65
Milk:buffer	1:1	1:1
HCl/NaOH qs	pH 5.7	pH 5.7
pH	5.7	5.7
Osmolarity (mOsm/kg)	340	240
Buffering Capacity (mEq/L/ ΔpH)	15	15

- 1058
- 1059 (a) *Pnc-FeSSGF – pediatric fed-state gastric media representative of neonates (0-28 days) fed cow's*
- 1060 *milk-based formula*
- 1061 (b) *Pns-FeSSGF – pediatric fed-state gastric media representative of neonates (0-28 days) fed soy-*
- 1062 *based formula*

1063

1064

1065 Table VII: Pediatric Fasted-State Simulated Intestinal Fluids (P-FaSSIF)

Component	P-FaSSIF-50% ^a	P-FaSSIF-150% ^b
Sodium Taurocholate (mM)	1.5	4.5
Lecithin (mM)	0.1	0.3
Maleic acid (mM)	19.12	19.12
Sodium hydroxide (mM)	34.8	34.8
Sodium Chloride (mM)	68.62	68.62
pH	6.5	6.5
Osmolarity (mOsm/kg)	180	180
Buffering Capacity (mEq/L/ ΔpH)	10	10

- 1066
- 1067 (a) P-FaSSIF-50% – pediatric fasted-state intestinal media formulated with bile salt concentrations
- 1068 50% (i.e. 1.5mM) of adult levels
- 1069 (b) P-FaSSIF-150% – pediatric fasted-state intestinal media formulated with bile salt concentrations
- 1070 150% (i.e. 4.5 mM) of adult levels
- 1071
- 1072

1073 Table VIII: Pediatric Fed-State Simulated Intestinal Fluids (P-FeSSIF)

Component	Pnb-FeSSIF ^a	Pnc-FeSSIF ^b	Pi-FeSSIF ^c
Sodium Taurocholate (mM)	2.5	2.5	7.5
Lecithin (mM)	0.5	0.5	1.5
Glyceryl monooleate (mM)	5	6.65	5
Sodium oleate (mM)	0.8	1.06	0.8
Maleic acid (mM)	55.02	55.02	55.02
Sodium hydroxide (mM)	81.65	81.65	81.65
Sodium Chloride (mM)	95	111.73	107.35
pH	5.8	5.8	5.8
Osmolarity (mOsm/kg)	300	330	330
Buffering Capacity (mEq/L/ ΔpH)	25	25	25

- 1074
- 1075 (a) *Pnb-FeSSIF – pediatric fed-state intestinal media representative of neonates (0-28 days) fed breast*
- 1076 *milk*
- 1077 (b) *Pnc-FeSSIF – pediatric fed-state intestinal media representative of neonates (0-28 days) fed cow's*
- 1078 *milk-based formula*
- 1079 (c) *Pi-FeSSIF – pediatric fed-state intestinal media representative of infants (1-12 months) fed cow's*
- 1080 *milk-based formula*

1081

1082

1083 Table IX: Predictive performance of Mithani et al.'s (17) equations at characterizing compound specific
1084 solubility changes in pediatric media

Compound	RMSE ^a
Dapsone (logP = 0.97)	8.5%
Griseofulvin (logP = 2.18)	3.7%
Carbamazepine (logP = 2.45)	16.9%
Phenytoin (logP = 2.47)	10.9%
Spironolactone (logP = 2.78)	17.4%
Indomethacin (logP = 4.27)	39.1%
Fenofibrate (logP = 5.3)	28.5%

1085

1086 (a) Root mean square error (RMSE) was tabulated based on Eq 4. Only pediatric media formulated
1087 with bile salts (i.e. P-FaSSGF, P-FaSSIF, and P-FeSSIF) were included in the assessment.

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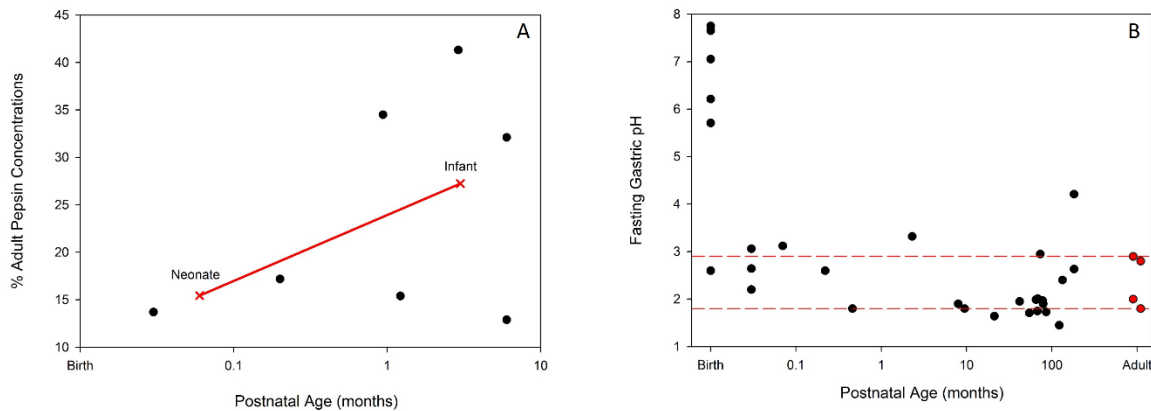
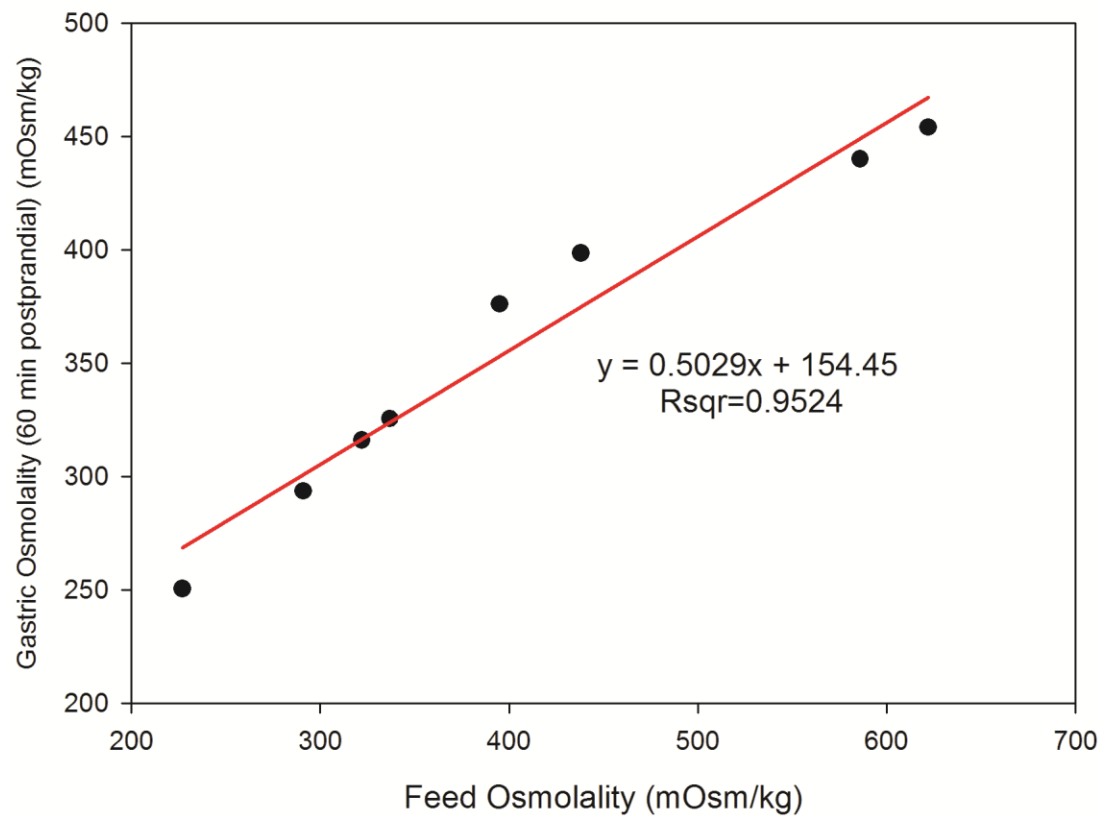


Figure 1: (A) Fasting gastric pepsin concentrations amongst pediatrics (21, 22, 25, 69) is expressed as a percentage of adult values (23, 25, 70). Investigations where pepsin concentrations were quantified over a specific age range without denoting the group's mean age were graphically depicted as the middle of the age range. Average (mean) values pertaining to neonates (0-28days) and infants (1m-12m) are illustrated for reference (red – x's). (B) Fasting gastric pH amongst pediatrics (black circles) is depicted as the central tendency, either mean or median, from separate investigations (18-20, 27, 38, 71-88). Studies where gastric pH values was quantified over specific age range without denoting the group's mean age were graphically depicted using the middle of the age range. Adult data is depicted by mean pH values from separate studies, as summarized by Di Maio and Carrier (26). Dashed reference lines correspond to the maximum and minimum mean pH values observed within the presented adult studies.



1103

1104 Figure 2: Neonatal gastric osmolality 60 minutes post-meal expressed as a function of feed osmolality.

1105 Data (black circles) represent average gastric osmolality values recorded amongst neonatal subjects as

1106 described by Billeaud et al (36). A linear regression model (red line) was fit to the data.

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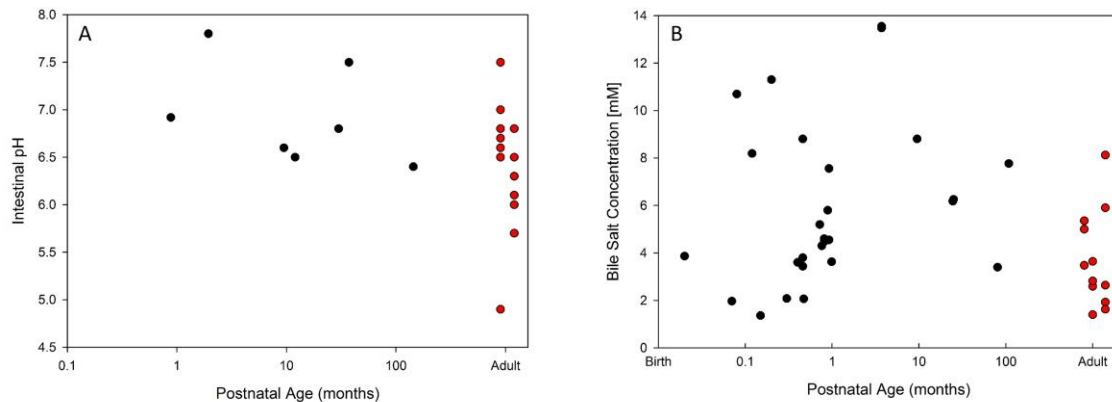
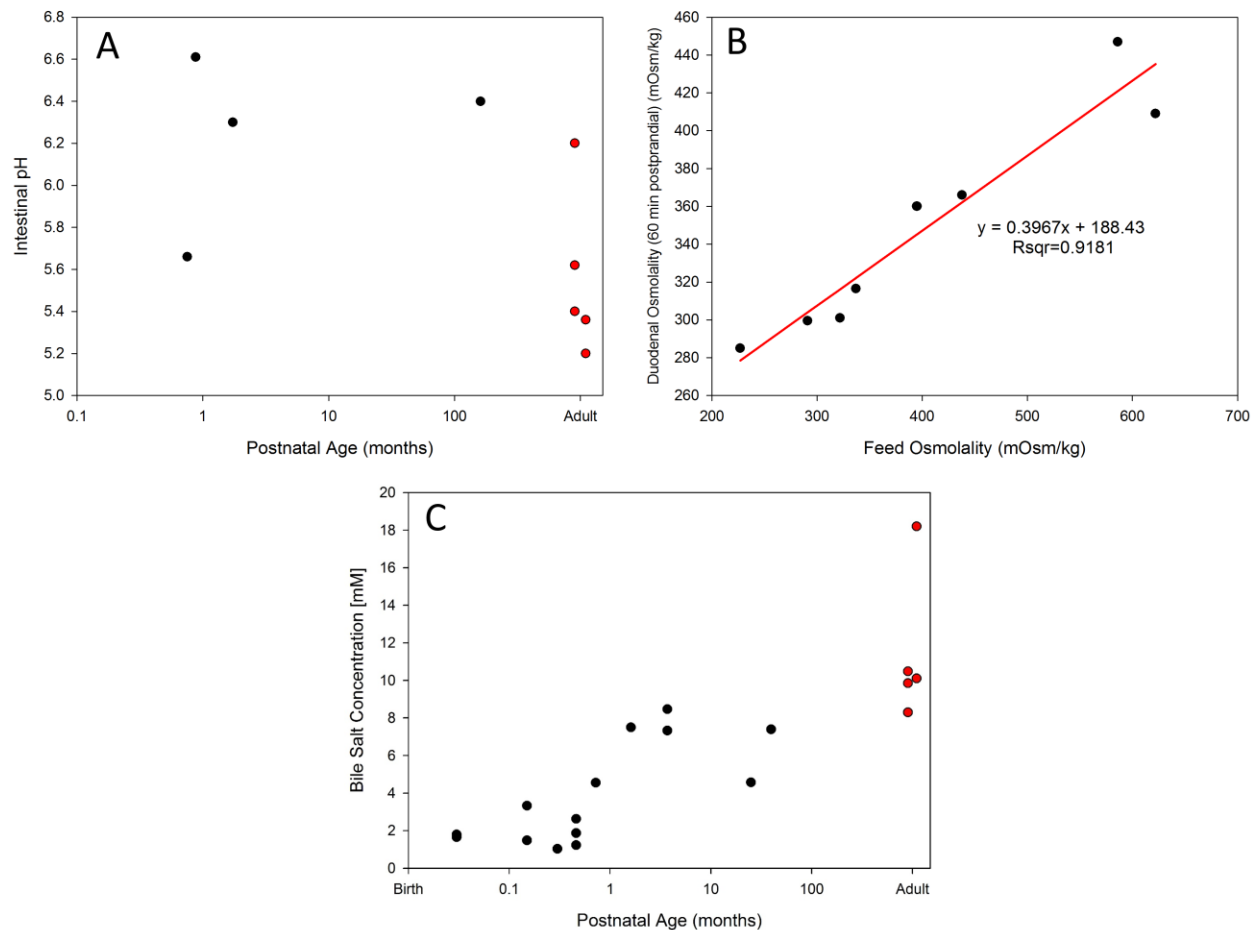


Figure 3: (A) Pediatric fasting intestinal pH (black circles) is depicted as the central tendency, either mean or median, from separate investigations (20-22, 89-92). Studies where pH was summarized over a specific age range without denoting the group's mean age were graphically depicted using the middle of the age range. The majority of data was derived from distal duodenum, though studies which included sampling sites from the proximal jejunum were also included. Adult duodenal bile acid concentrations (red circles) are depicted as mean values from separate studies, as summarized by Fuchs and Dressman (39). (B) Fasting duodenal bile salt concentrations amongst pediatrics (black circles) are depicted as the central tendency, either mean or median, from separate investigations (43, 44, 93-101). Studies where bile acids were summarized over a specific age range without denoting the group's mean age were graphically depicted using the middle of the age range. Adult duodenal bile acid concentrations (red circles) are depicted as mean values from separate studies, as summarized by Fuchs and Dressman (39).



1121
 1122 Figure 4: (A) Fed-state duodenal pH from separate pediatric investigations (black circles) is depicted by
 1123 the central tendency, either mean or median (90, 102-104). Studies where pH was summarized over a
 1124 specific age range without denoting the group's mean age were graphically depicted using the middle of
 1125 the age range. Adult pH values (red circles) are presented as the central tendency (mean or median)
 1126 from separate investigations (33, 34, 40, 90, 105). (B) Neonatal duodenal osmolality 60 minutes post-
 1127 meal expressed as a function of feed osmolality. Data (black circles) represent average duodenal

1128 osmolality values recorded amongst neonatal subjects as described by Billeaud et al (36). A linear
1129 regression model (red line) was fit to the data. (C) Fed-state duodenal bile salt concentrations amongst
1130 pediatrics (black circles) is depicted as the mean from separate investigations (44, 96, 98, 99, 103, 106,
1131 107). Studies where data was summarized over a specific age range without denoting the group's mean
1132 age were graphically depicted using the middle of the age range. Adult duodenal bile acid
1133 concentrations (0.5-1hr postprandially) (red circles) are depicted as the mean value from the various
1134 publications (26, 33, 40, 108, 109).

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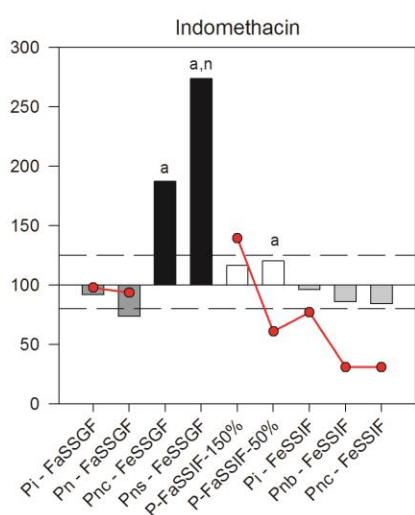
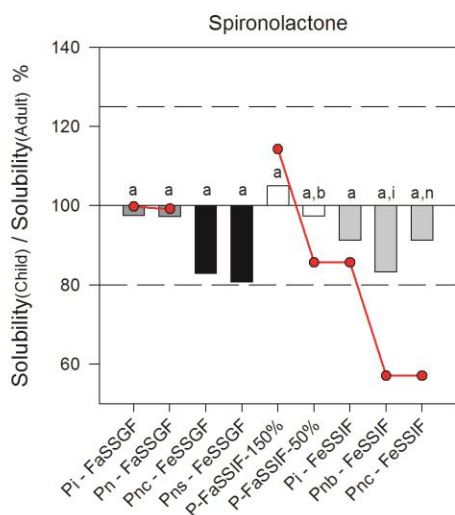
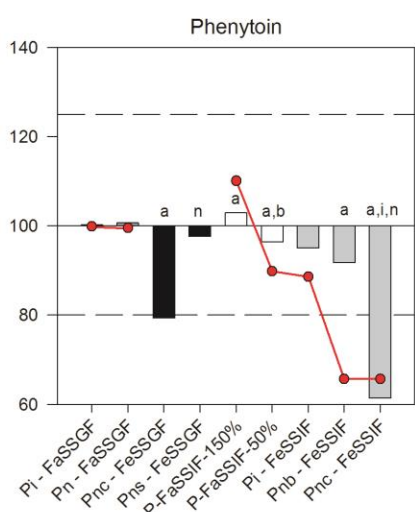
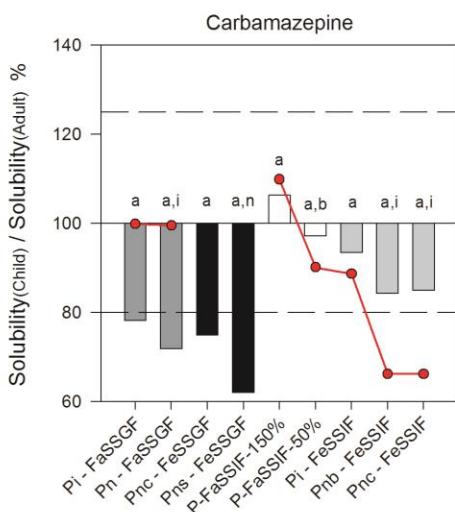
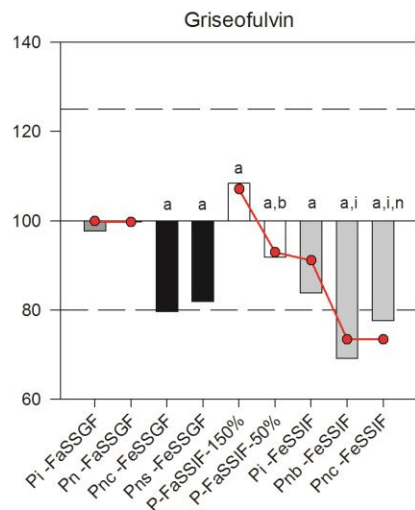
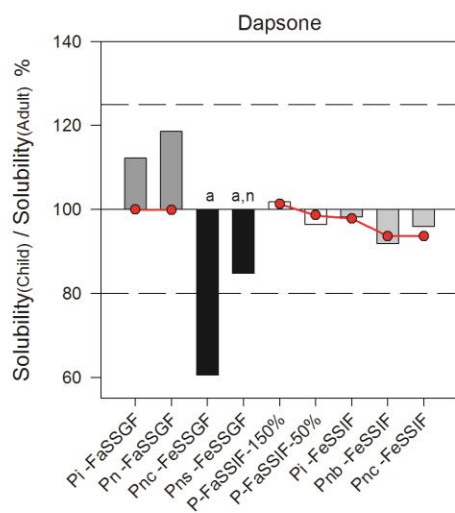
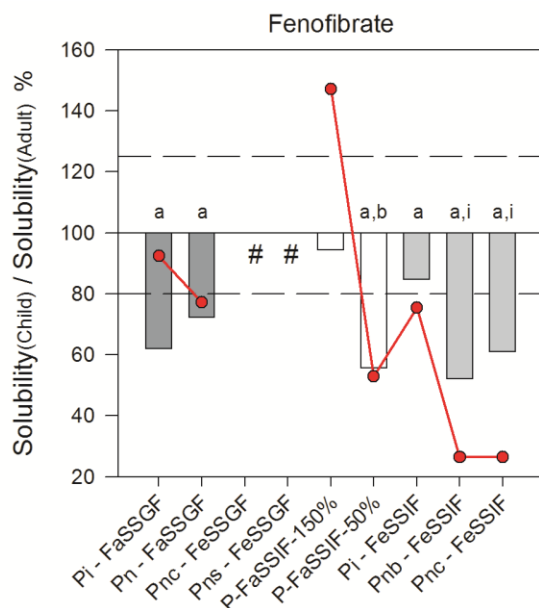


Figure 5: Measured solubility in age-specific biorelevant media expressed as the mean solubility ratio (bars) between each respective pediatric and adult media (i.e. $\text{Pi-FaSSGF} / \text{FaSSGF}_{\text{Adult}}$). Predicted solubility ratios due to differences in media bile acid content were calculated for P-FaSSGF, P-FaSSIF, and P-FeSSIF according Mithani et al.'s equations (red line, dots) (17). Dashed lines (---) characterizing the bioequivalence criterion (80-125%) are displayed for reference. Media are denoted as follows: Pi-FaSSGF (Infant FaSSGF), Pn-FaSSGF (Neonate FaSSGF), Pnc-FeSSGF (Neonate FeSSGF comprised of cow's milk-based formula), Pns-FeSSGF (Neonate FeSSGF comprised of soy-based formula), P-FaSSIF-150% (Pediatric FaSSIF comprised with 4.5 mM NaTc), P-FaSSIF-50% (Pediatric FaSSIF comprised with 1.5 mM NaTc), Pi-FeSSIF (Infant FeSSIF), Pnb-FeSSIF (Neonatal breast-fed FeSSIF), and Pnc-FeSSIF (Neonatal formula-fed FeSSIF). Statistically significant solubility differences ($p \leq 0.05$) compared to (a) adult media, (i) infant media, (n) neonatal media, and (b) P-FaSSIF-150% were depicted using the symbols indicated.



1151
 1152 Figure 6: Measured solubility in age-specific biorelevant media expressed as the mean solubility ratio
 1153 (bars) between each respective pediatric and adult media (i.e. Pi-FaSSGF / FaSSGF_{Adult}). Predicted
 1154 solubility ratios due to differences in media bile acid content were calculated for P-FaSSGF, P-FaSSIF, and
 1155 P-FeSSIF according Mithani et al.'s equations (red line, dots) (17). Dashed lines (---) characterizing the
 1156 bioequivalence criterion (80-125%) are displayed for reference. For a description of media abbreviations
 1157 and symbols (a,b,n,i), see the footnote to Figure 5.

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